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# Molecular diagnostics and chemical analysis for assessing biodegradation of polychlorinated biphenyls in contaminated soils

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## SUMMARY

The microbial populations in PCB-contaminated electric power substation capacitor bank soil (TVA soil) and from another PCB-contaminated site (New England soil) were compared to determine their potential to degrade PCB. Known biphenyl operon genes were used as gene probes in colony hybridizations and in dot blots of DNA extracted from the soil to monitor the presence of PCB-degrading organisms in the soils. The microbial populations in the two soils differed in that the population in New England soil was enriched by the addition of 1000 p.p.m. 2-chlorobiphenyl (2-CB) whereas the population in the TVA capacitor bank soil was not affected. PCB degradative activity in the New England soil was indicated by a 50% PCB disappearance (gas chromatography), accumulation of chlorobenzoates (HPLC), and $^{14}CO_2$ evolution from $^{14}$C-2CB. The PCB-degrading bacteria in the New England soil could be identified by their positive hybridization to the *bph* gene probes, their ability to produce the yellow *meta*-cleavage product from 2,3-dihydroxybiphenyl (2,3-DHB), and the degradation of specific PCB congeners by individual isolates in resting cell assays. Although the TVA capacitor bank soil lacked effective PCB-degrading populations, addition of a PCB-degrading organism and 10000 p.p.m. biphenyl resulted in a >50% reduction of PCB levels. Molecular characterization of soil microbial populations in laboratory scale treatments is expected to be valuable in the design of process monitoring and performance verification approaches for full scale bioremediation.

## INTRODUCTION

Major technical difficulties in the field implementation of bioremediation include adequate site characterization, and process monitoring and performance verification before and during implementation of any hazardous waste treatment process [16]. The most common method for assessing degradative activity is to determine the residual concentrations of the contaminants in treatability tests and/or any resulting degradative intermediates or products. In studies of biodegradation of polychlorinated biphenyls (PCBs), the compounds commonly monitored are residual PCB [4,17,18,38], PCB metabolites including chlorobenzoic acids [11,17,18], and $^{14}CO_2$ [7,13,21]. Although these chemical analyses are effective in laboratory scale studies, they are often difficult to apply in full scale bioremediation. Molecular diagnostics may be useful as an adjunct to these traditional analyses. Chemical methods for monitoring bioremediation indicate the results of biological activity, whereas molecular diagnostics can be used for the direct quantification of the responsible microbial populations, degradative genes, and gene transcripts (activity) under field conditions [32]. These techniques are expected to be useful in elucidating the factors limiting biodegradation at particular sites.

It is generally accepted that PCBs are cometabolized aerobically by the biphenyl degradative pathway. The genetic operon consits of the *bph*ABCD genes, and has been isolated from both Gram-negative [1,14,19,24,28,35,36], and Gram-positive bacterial species [3,30]. In some Gram-negative microorganisms capable of PCB degradation, the *bph* operon appears to be conserved [15,41]. The *bph* operons from *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas* LB400 have been sequenced and show over 95% sequence homology [9,10,22,37,41]. The *bph* operon from *Pseudomonas testosteroni* B-356 was reported to have a 17% homology as determined by DNA hybridization analysis with the *bph* genes from *Pseudomonas* LB400 [1]. The *bph* operons from several other organisms, including *Pseudomonas paucimobilis* Q1 [36] and *Rhodococcus globerulus* P6 [3], show little homology to the *bph* genes from *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas* LB400 [3,36].

The availability of gene probes from several PCB-degrading strains and the presence of a detailed body of literature on the genetics of the *bph* operon provide the opportunity for the direct examination of the PCB-degradative populations in contaminated soils. Gene probes have been used to isolate 4-chlorobiphenyl-degrading bacteria [31] and to demonstrate the presence of PCB-degrading bacteria in contaminated sediments and soils [8,39]. However, comparison of these results with traditional chemical analyses is necessary for the devel-

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opment and acceptance of molecular diagnostics for site evaluation, process monitoring, and performance verification in full scale bioremediation.

Many localized point sources of PCB contamination exist from the use of manufactured products and equipment containing PCBs. In the electric utility industry, use of PCBs as dielectric fluids in capacitors and transformers has resulted in widespread but localized contamination of soils with PCBs. PCB contamination at power generation facilities and electrical substations resulted as aged equipment leaked, failed or ruptured during peak overloads. The potential for microbial degradation as an option for treating the contaminated substation soils is being considered because of the localized nature of PCB contamination at electrical substations, cost of alternative remediation strategies, and the desire to keep substations in service. In addition, in situ PCB biodegradation has been demonstrated in Hudson river sediments [11,17] and in soil microcosms [4,18,21,38]. In collaboration with the Tennessee Valley Authority, an investigation was initiated to characterize substation sites chemcially and microbiologically for bioremediation potential, to develop bioremediation technologies, and to develop molecular diagnostic techniques using known biphenyl operon genes as DNA probes for process monitoring and performance verification. For comparison, experiments were also performed on a PCB-contaminated soil from a site in New England.

## MATERIALS AND METHODS

### *Soil samples*

The TVA soil sample was obtained from an electric power substation in Chattanooga, TN as previously described [27]. The site is stratified in three layers: gravel, interface and soil. The vertical distribution of PCB is limited to the gravel and interface layers with the greatest concentration in the interface level. The contaminated sample used in these studies is a composite taken from the interface level from one of the capacitor banks at the transformer substation. The capacitors originally contained Aroclor 1242 (Westinghouse), although the PCB extracted from the soil resembles weathered Aroclor 1248 with several of the less chlorinated congener peaks absent (Table 1). The PCB concentration in the composite sample was 40 p.p.m. (± 2 p.p.m.) as calculated from comparison with the peak areas from a 1 p.p.m. Aroclor 1248 standard and corresponding weight percent values for each congener in Aroclor 1248.

A PCB-contaminated sandy soil from New England was a composite sample containing 144 p.p.m. PCB (± 9 p.p.m.) as calculated by comparison with peak areas and weight percent values for 1 p.p.m. Aroclor 1242 [33]. This soil contained more of the less chlorinated PCB congeners than the TVA soil (Table 1, Fig. 1).

### *2-Chlorobiphenyl enrichments*

2-chlorobiphenyl (1000 p.p.m.) enrichments were performed in the laboratory on individual soil slurries consisting of 30 g of soil and 150 ml PAS medium [5]. Flasks were incubated at 25 °C at 100 r.p.m. and sampled over an 8-week period. The genetic composition of the microbial community was determined from total colony forming units followed by colony hybridization with the *bph* gene probes and by direct DNA extraction of the soil slurries followed by slot blot hybridization with DNA gene probes. $^{14}$C-2CB mineralization assays, ether extraction of PCB residues and analysis by congener-specific capillary gas chromatography, and HPLC analysis of aqueous filtrates for chlorobenzoic acids were used to measure the degradative activity of the microbial populations in the soils.

### *Bacterial enumeration and determination of biphenyl genotype and phenotype*

At each time point 1 ml of the soil slurries and 9 ml of PAS medium were mixed by vortexing for 30 s. Serial dilutions were plated on 1/4 strength R2A agar medium (Difco Laboratories, Detroit, MI, USA) and incubated at 25 °C for 3 days. Colony forming units (CFU) were reported as the average of at least three plates.

For colony hybridizations, bacterial lifts were made onto Biotrans nylon membranes following manufacturer protocols (ICN Biomedicals, Inc., Costa Mesa, CA, USA). DNA hybridizations were performed at 65 °C following the Genius system protocols (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). The following DNA fragments from cloned biphenyl genes were used as DNA probes: pKF707 *bph*BC (2.0 kb *Pst*I) [14,37]; C14-15 *bph*AB (4.0 kb *Cla*I-*Bam*HI) [26,34]; and pDA251 *bph*BC (3.2 kb *Sma*I) [2]. Digoxigenin-labelled DNA probes were prepared and detected following manufacturer protocols as previously described (25,27). In the time course experiment, the percentage of *bph*-positive bacterial colonies in the microbial community was determined by colony hybridization of the serial dilution plates with the pKF707 *bph*BC gene probe. *Bph*-CFU were reported as the average of three filters per time point.

For correlation of gene probes with the biphenyl phenotype (2,3-dihydroxybiphenyl meta-cleavage assay) at the individual colony level, single colonies from several time points in both soil samples were streaked onto 1/2 strength R2A agar plates and replicated onto a series of four 1/2 strength R2A agar plates. PCB-degradative activity of individual isolates was determined by spraying the surface of agar plates containing bacterial colonies with a 0.1% solution of 2,3-dihydroxybiphenyl in ether. A positive reaction, produced as a result of the meta-cleavage of 2,3-dihydroxybiphenyl (Waco Chemicals USA, Inc., Richmond, VA, USA) yielded a yellow color in minutes after the etheral spray [28].

### *Direct DNA extraction*

Total DNA was extracted from 5-ml soil slurry samples following a modified method of Ogram and Sayler [29]. To each 5-ml soil slurry sample, 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing 1% sodium pyrophosphate and 0.1% polyvinylpyrrolidone, 20 μl 0.5 M EDTA, and 0.5 ml 10% sodium dodecyl sulfate were added. The samples were mixed by inversion and incubated at 70 °C for 20 min. Samples were cooled to 37 °C, 25 μl of a 12.5 mg ml$^{-1}$ proteinase K stock solution were added, and the samples were incubated for 30 min at 37 °C. An equal volume of chloro-

TABLE 1

Percentage degradation of PCB congeners by biphenyl-utilizing bacterial strains in resting cell assays and in soil treatment experiments with PCB-contaminated soils

| Peak no. | Congener[a] | % Degradation NE2-1[b] | New England soil | | TVA soil | |
|---|---|---|---|---|---|---|
| | | | Initial mg kg$^{-1}$ [c] | % Degradation in situ[d] | Initial mg kg$^{-1}$ [e] | % Degradation with GG4202[f] |
| 1 | 2,5,2′ | >99 | 0.33 | >99 | np* | np |
| 2 | 2,4,2′/4,4′ | 95 | 4.20 | >85 | 0.08 | 20 |
| 3 | 2,3,6/2,6,3′ | 60 | 2.94 | 60 | 0.19 | 40 |
| 4 | 2,5,4′ | >99 | 6.67 | >99 | np | np |
| 5 | 2,4,4′ | 80 | 13.63 | 60 | 0.87 | 40 |
| 6 | 2′,3,4/2,5,2′,6′ | >99 | 5.50 | 95 | 0.17 | 75 |
| 7 | 2,3,4′/2,4,2′,6′ | >95 | 2.8 | 80 | 0.19 | 40 |
| 8 | 2,3,6,2′ | 55 | 1.59 | 55 | 0.19 | 55 |
| 9 | 2,3,2′,6′ | >99 | 0.19 | >85 | np | np |
| 10 | 2,5,2′,5′ | >99 | 7.12 | 80 | 0.34 | 95 |
| 11 | 2,4,2′,5′ | >99 | 6.66 | 95 | 1.19 | 85 |
| 12 | 2,4,2′,4′ | 85 | 1.66 | 70 | 0.50 | 50 |
| 13 | 2,4,5,2′ | 90 | 1.04 | 85 | 0.34 | 85 |
| 14 | 2,3,2′,5′ | >99 | 4.5 | 95 | 1.79 | 90 |
| 15 | 3,4,4′/2,3,2′,4′ | 70 | 1.68 | 50 | 1.24 | 70 |
| 16 | 2,3,4,2′/2,3,6,4′/2,6,3′,4′ | 40 | 4.98 | 30 | 2.36 | 40 |
| 17 | 2,3,2′,3′ | >99 | 0.90 | >95 | 1.64 | 90 |
| 18 | 2,4,5,4′ | 0 | 2.82 | 15 | 1.86 | 0 |
| 19 | 2,5,3′,4′ | 100 | 6.85 | 85 | 1.23 | 80 |
| 20 | 2,4,3′,4′/2,3,6,2′,5′ | 25 | 9.18 | 25 | 5.21 | 30 |
| 21 | 2,3,6,2′,4′ | 35 | 0.53 | 50 | 0.59 | 50 |
| 22 | 2,3,3′,4′/2,3,4,4′ | 20 | 4.39 | 25 | 4.35 | 20 |
| 23 | 2,3,6,2′3′/2,3,5,2′,5′ | 60 | 2.82 | 50 | 0.78 | 80 |
| 24 | 2,3,5,2′,4′/2,4,5,2′,5′ | 65 | 7.16 | 40 | 1.45 | 85 |
| 25 | 2,4,5,2′,4′ | 25 | 2.68 | 25 | 0.91 | 55 |
| 26 | 2,4,5,2′,3′/2,3,5,6,2′,6′ | 25 | 2.44 | 40 | 0.96 | 60 |
| 27 | 2,3,4,2′,5′ | 25 | 2.13 | 65 | 1.31 | 65 |
| 28 | 2,3,4,2′,4′ | 15 | 1.36 | 35 | 0.48 | 0 |
| 29 | 2,3,6,3′,4′/3,4,3′,4′ | 15 | 7.76 | 30 | 2.19 | 30 |
| 30 | 2,3,4,2′,3′ | 55 | 1.08 | 75 | 0.60 | 40 |
| 31 | 2,3,6,2′,4′5′/2,4,5,3′,4′ | 0 | 14.7 | 0 | 2.25 | 0 |
| 32 | 2,3,4,3′,4′/2,3,4,2′,3′,6 | 0 | 4.53 | 0 | 2.13 | 0 |
| 33 | 2,3,4,2′,4′,5′/2,3,5,6,3′,4′ | nc** | nc | 0 | 0.84 | 0 |

[a] Aroclor 1248 peak numbers and corresponding congeners are presented following Bedard et al. [6].

[b] % Degradation of PCB congeners by *B. gladioli* NE2-1 in resting cell assays (10 p.p.m. Aroclor 1242) for 48 h. Congeners in Aroclor 1242 were aligned to congeners in Aroclor 1248 following Bedard et al. [6].

[c] PCB concentration (mg kg$^{-1}$) in New England soil determined by comparison of peak areas with 1 p.p.m. Aroclor 1242 and published weight percents of each congener in Aroclor 1242 [33].

[d] % Degradation of in situ PCBs in New England soil after the addition of 1000 p.p.m. 2-CB. % Degradation was determined by comparison of the peak areas at 0 and 4 weeks incubation.

[e] PCB concentration (mg kg$^{-1}$) in TVA soil determined by comparison of peak areas with 1 p.p.m. Aroclor 1248 and weight percents of individual congeners present in Aroclor 1248.

[f] % Degradation of in situ PCBs in TVA soil 32 days after the addition of *A. eutrophus* GG4202 and 10 000 p.p.m. biphenyl. % Degradation determined by comparison of peak areas with uninoculated controls.

* np = congener not present in the soil sample.

** nc = not calculated because this peak is not present in the Aroclor 1242 standard.

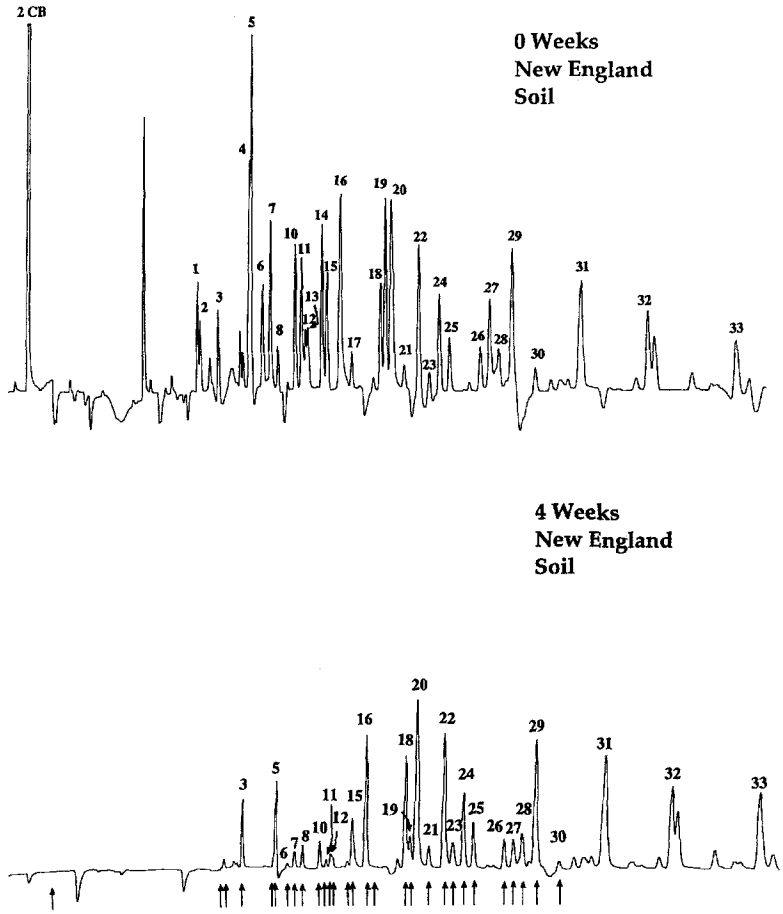



Fig. 1. Biodegradation of PCBs in New England soil amended with 2-chlorobiphenyl, gas chromatograms of soil extracts at 0 weeks and 4 weeks of incubation. Arrows represent >20% removal of individual PCB congeners. Peak numbers correspond to congeners present in Aroclor 1248.

form: isoamyl alcohol (24:1) was added and the samples were mixed by inversion. After centrifugation for 10 min at 7700 × *g*, the upper aqueous phase was removed to a clean 30-ml plastic centrifuge tube. The supernatant was precipitated at room temperature for 30 min with an equal volume of isopropanol, centrifuged at 7700 × *g* for 30 min, and the resulting pellet was washed with 70% ethanol. Pellets were resuspended in 500 μl TE (10 mM Tris, 1 mM EDTA pH 8.0). Particulate material was pelleted by centrifugation and the supernatant was precipitated again with isopropanol The pellets were washed with 70% ethanol, dried and resuspended in 100 μl of TE buffer. For dot blots, dilutions of the DNA samples were made and denatured with an equal volume of 0.5 M NaOH. Dilutions of denatured DNA samples (2 μl) were spotted onto Biotrans Nylon membranes (0.2 μm) (ICN Biomedicals, Inc., Costa Mesa, CA, USA) and air dried. The amount of sample per spot was equivalent to 1 μl, 0.3 μl and 0.1 μl of the DNA extract. Filters were baked and hybridized with digoxigenin DNA probes as described above. DNA hybrids were detected by the chemiluminescent method (BMB manufacturer protocols). DNA was quantified on Visage 110 Bioimager (BioImage Products, Ann Arbor, MI, USA) by comparison with *bph*BC DNA standards. The number of *bph*BC sequences was calculated assuming 1 ng of DNA contains $4.6 \times 10^8$ *bph*BC genes (2.0 kb in length).

*$^{14}C$ mineralization assays*

$^{14}C$-$CO_2$ from $^{14}C$-2 chlorobiphenyl (2-CB) (11.2 mCi mmol$^{-1}$, 99% purity, Pathfinder Laboratories, St Louis, MO, USA) biodegradation was assayed by experimentally treating 2.5-ml subsamples of soil slurries with 200 000 d.p.m. uniformly labeled $^{14}C$-2-CB in 3 μl acetone. For each time point, three killed control vials and three experimental vials were incubated for 2 days and assayed following previously published methods [32]. $^{14}CO_2$ evolved in each vial was trapped in 0.5 ml of 0.5 N NaOH which was then added to 1 ml water and 10 ml Beckman Ready Safe$^{Tm}$ (Beckman Instruments, Inc., Fullerton, CA, USA) scintillation fluid. Radioactive counts were determined using a Beckman liquid scintillation counter (Model LS3801). Values for the killed control vials were subtracted as background in order to calculate the percentage $^{14}C$-2-CB mineralized.

*Resting cell assays*

Individual soil isolates that probed positive with the *bph* probes were tested for growth in PAS medium amended with

biphenyl. The ranges of PCB congeners metabolized by one representative isolate from the New England soil was determined using a 48-h resting cell assay with 10 p.p.m. Aroclor 1242 [6,26,27].

*PCB extraction and gas chromatography*

At each time point, triplicate 5-ml soil slurry samples were extracted with ether as previously described [26,27]. PCB in resting cell assays and soil treatment experiments (5-ml slurry subsamples) were extracted with ether and quantified by capillary gas chromatography as previously described [26,27]. The ether extract was diluted in hexane and purified with silica gel. Dilutions were made so that approximately 1–2 p.p.m. of PCBs from time zero soil slurry samples were injected onto the gas chromatograph. The same dilutions were used throughout the experiment. Relative concentrations of PCB congeners in the ether-hexane extracts were determined using a Shimadzu (Kyoto, Japan) model GC-14A gas chromatograph equipped with an AOC-14 auto injector, and an electron capture detector and a split/splitless injector, both maintained at 300 °C. A DB-1 capillary column (30 m × 0.25 mm i.d., J & W Scientific, Inc., Folsom, CA, USA) was used with nitrogen as the carrier (0.88 ml $min^{-1}$) and make-up (35 ml $min^{-1}$) gas. Injection of 1 or 2 $\mu$l was performed using the splitless mode. The column oven temperature was held at 40 °C for 2 min, raised to 80 °C at a rate of 10 °C $min^{-1}$, and then to 225 °C at 6 °C per min and held for 45 min [6].

For resting cell assays, individual congeners were quantified and identified by comparison of congener profile with those reported by Bedard et al. [6] using peak number 41 (congeners 2,3,4,3′,4′ and 2,3,4,2′,3′,6′) as an internal standard. For soil treatment procedures using Aroclor 1248, peak 32 (also congeners 2,3,4,3′,4′ and 2,3,4,2′,3′,6′) was used as an internal standard. The percent degradation was calculated by comparison with killed controls in the resting cell assay, and by comparison with the initial concentrations in the soil treatment studies. Percent degradation was rounded to the nearest 5% and degradation of less than 15% was considered as zero [6].

Spike and recovery experiments in contaminated soil and uncontaminated control soil using Aroclor 1248 (50 p.p.m.), with 2 p.p.m. 4-CB added after extraction as an internal standard, indicated an average extraction efficiency of 90% for all congeners, with no individual congener less than 80% [27].

*HPLC analysis*

Slurry samples (3 ml) were filtered through 0.45-$\mu$m PTFE filters (Gelman). The aqueous metabolites were separated on a Supelcosil LC-18 column (Supelco, Bellefonte, PA, USA) by eluting with a linear gradient (5% $min^{-1}$, 2 ml $min^{-1}$ flow rate) from 100% water (pH 2.5) to 60% acetonitrile: 40% water and holding for 4 min. Detection was by a photodiode array detector (Model LC-235, Perkin-Elmer Corp., Groton, CT, USA) at a wavelength of 255 nm.

*Biodegradation of PCB in TVA soil*

Three treatments using 10 g TVA soil samples plus 50 ml PAS were prepared as follows: 1) no additional amendments; 2) addition of 10 000 p.p.m. biphenyl; and 3) 10 000 p.p.m. biphenyl plus $5.2 \times 10^5$ cells $ml^{-1}$ slurry of *Alcaligenes eutrophus* GG4202. *A. eutrophus* GG4202 was isolated by IT corporation by biphenyl enrichment from a PCB-contaminated surface soil containing approximately 1700 p.p.m. PCBs. *A. eutrophus* GG4202 is capable of degrading a large number of PCB congeners in an Aroclor 1242 resting cell assay and hybridizes with the pKF707 gene probe [27]. After 32 days of incubation, three 5-ml slurry samples were extracted with ether for GC analysis as described above. The percent degradation was calculated by comparison of the amount of residual PCBs in the biphenyl treatment and biphenyl + *A. eutrophus* treatment with the untreated control.

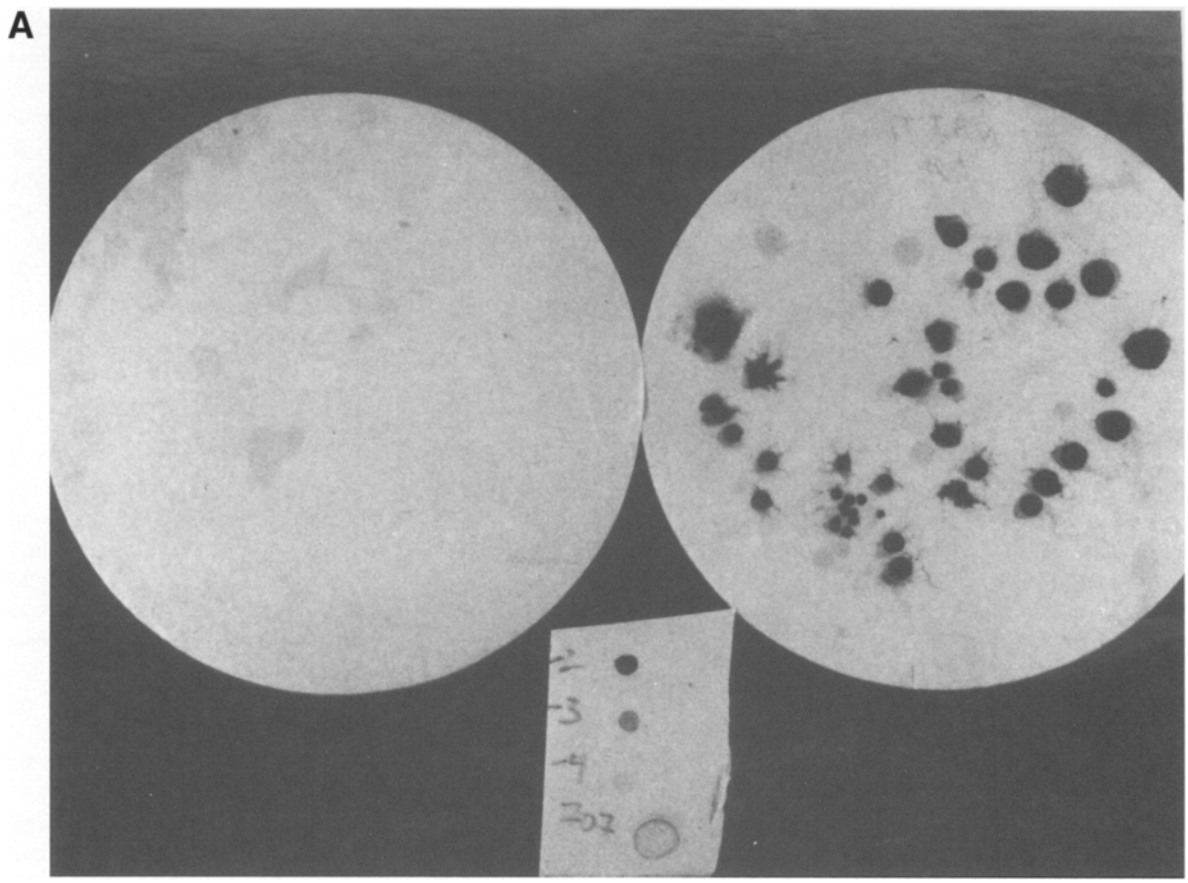
## RESULTS

*2-Chlorobiphenyl enrichment of New England soil*

Chemical analysis indicated that amendment of New England soil with 2-CB stimulated PCB degradation. After 4 weeks of incubation a wide range of PCB congeners exhibited substantial disappearance (Table 1, Fig. 1) and the added 2-CB was completely removed. The amount of PCBs in the New England soil was reduced from a starting concentration of 144 p.p.m. (± 9 p.p.m.) (wt PCB/wt soil) to 61 p.p.m. (± 6 p.p.m.). The amount of $^{14}$C-2CB mineralization after 2 days was used as a measure of bacterial activity at several time points in the experiment. The amounts of $^{14}$C-2CB mineralized in a 2-day assay when the soil slurry was sampled at 0, 2, and 8 weeks were 0, 60, and 14 percent, respectively. This indicated that the bacterial activity increased at 2 weeks and then declined at 8 weeks. After 1 week of incubation, degradation products in aqueous filtrates from the soil slurries were tentatively identified as 2-chlorobenzoic acid (retention time, rt = 9.631 min) and either 3-chlorobenzoic acid or 4-chlorobenzoic acid (rt = 10.644 min). Other unidentified peaks were apparent. After 4 weeks of incubation the chlorobenzoic acid peaks were greatly reduced, whereas two other unidentified peaks were not removed even after 8 weeks.

Molecular analysis indicated that amendment of New England soil with 2-CB stimulated growth of PCB-degradative organisms. Colony hybridizations and dot blot analysis of DNA extracts from the soil using pKF707 *bph*BC yielded positive results (Fig. 2). At time 0, no positive hybridization signals were seen in the dot blot hybridization of DNA soil extracts. Therefore, the calculated number of DNA sequences was less than the minimum detection limit of $1.4 \times 10^9$ sequences per gram of soil (Fig. 3(A)). For the 0 week time point, it was possible to calculate the number of *bph*BC positive colonies (*bph* CFU) from colony hybridization as $9 \times 10^2$ colonies per gram of soil (Fig. 3(A)). The number of *bph* CFU calculated by colony hybridization and the number of DNA sequences calculated from DNA extracts on dot blots followed the same relative pattern, with the number of DNA sequences being 2–3 orders of magnitude higher for soil DNA extracts than the *bph* CFU (Fig. 3(A)).

Time course comparisons of PCB concentrations (2-CB and 2,3,2′,5′-tetrachlorobiphenyl) and the microbial populations (total CFU, *bph* CFU, and *bph* Seq) indicate a clear relation-



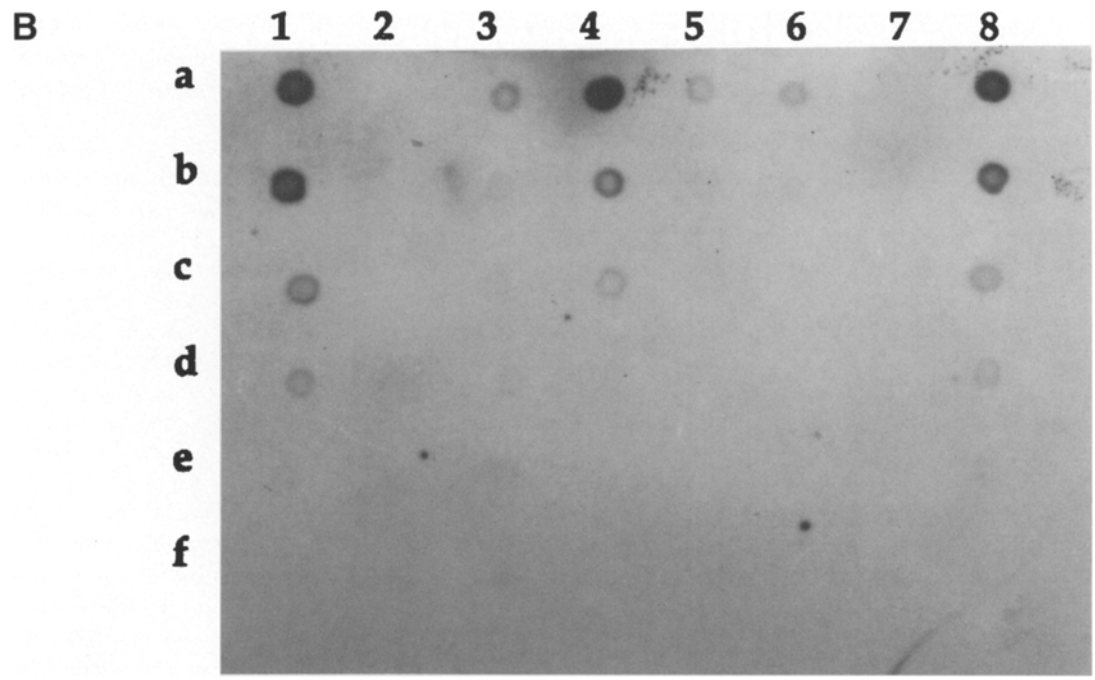




Fig. 2. (A) Colony hybridization of bacteria from the TVA (left) and New England (right) soils after 1 week incubation with 2-chlorobiphenyl. The pKF707 *bph*BC fragment was used as the gene probe. (B) Dot blot hybridization of DNA extracted from New England and TVA soil with the pKF707 *bph*BC gene probe. Lanes 1 and 8 pKF707 DNA standards (*bph*BC DNA concentrations 30 ng, 10 ng, 3 ng, 1 ng, 0.3 ng), Lane 2a–c NE-T0, Lane 3a–c NE-T1, Lane 4a–c NE-T2, Lane 5a–c NE-T4, Lane 6a–c NE-T8, Lane 7 Lambda DNA (DNA concentrations 30 ng, 10 ng, 3 ng, 1 ng), Lanes 2d–f TVA-T0, Lanes 3d–f TVA-T1, Lanes 4d–f TVA-T2, Lanes 5d–f TVA-T4, Lanes 6d–f TVA-T8. Volumes of DNA soil extracts added were 1 $\mu$l, 0.3 $\mu$l, 0.1 $\mu$l for rows a, b and c for New England soil samples and d, e, f for TVA soil samples.

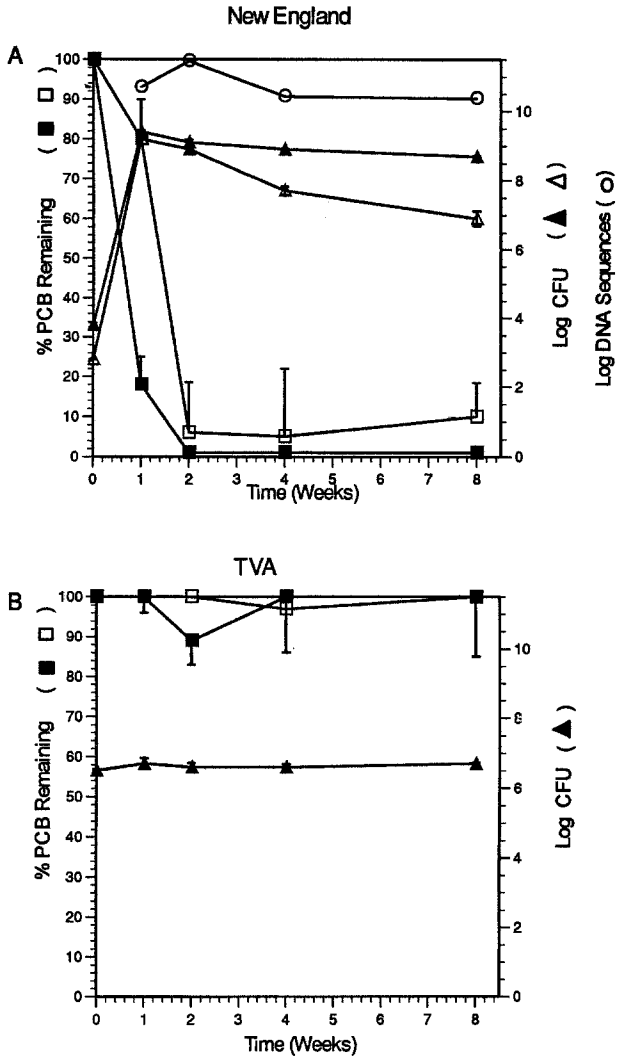



Fig. 3. Bacterial growth and degradation of PCBs after the addition of 2-CB (1000 p.p.m.) in (A) New England soil and (B) TVA soil. In the New England soil at 0 weeks the number of DNA sequences determined by hybridization of DNA extracts with the pKF707 *bph*BC gene probe was below the detection limit. No Bph CFU or Bph sequences were detected in the TVA soil. -■- 2-CB, -□- 2,3,2′,5′ CB, -▲- CFU, -△- Bph CFU, -○- Bph sequences.

ship between growth of PCB-degradative organisms and PCB degradation (Fig. 3(A)). This is also indicated by mineralization of $^{14}$C-2CB in slurry subsamples, and the accumulation of aqueous metabolites in soil slurry filtrates. The removal of PCB congeners did not continue after 2 weeks when greater than 99% of the 2-CB was removed.

The correlation between the DNA hybridization data and biphenyl phenotype (*meta*-cleavage product) was confirmed by picking a total of 205 colonies from time 0, time 1 and time 2. All individual colonies that probed positive with pKF707 *bph*BC also probed positive with C14-15 *bph*AB. Eighty six percent of colonies that probed positive with the pKF707 *bph*BC gene probe also produced the yellow metabolite from 2,3-dihydroxybiphenyl in the spray plate assay. The colonies that hybridized with pKF707 *bph*BC and C14-15 *bph*AB did not hybridize to the pDA251 *bph*C gene probe.

The bacterial community present at 1 and 2 weeks was dominated by a single genotype as evidenced by the high number of *bph*BC-positive colonies. The pattern of congener degradation by a representative soil isolate, NE2-1, in a resting cell assay was similar to the pattern of PCB degradation in the New England soil (Table 1). This strain was identified by fatty acid analysis (Microcheck, Inc., Northfield, VT 05663, USA) as *Burkholderia cepacia* (basonym: *Pseudomonas cepacia* [40]) and as *Burkholderia gladioli* (basonym: *Pseudomonas gladioli* [40]) using specific phenotypic tests [23].

*2-Chlorobiphenyl enrichment of TVA soil*

Chemical analysis incidated that amendment of TVA soil wtih 2-CB did not stimulate PCB degradation (Fig. 3(B)). No disappearance of 2-CB or any PCB congeners occurred. No $^{14}CO_2$ was evolved in the 2-day mineralization assays at T0, T4, and T8 with $^{14}$C-2CB amended slurry subsamples. Addition of 2-CB to TVA soil slurries did not result in an increase in the number of indigenous bacteria comparable to that observed in the New England soil (Fig. 3(B)). The pKF707 *bph*BC gene probe did not hybridize with the bacterial colonies during the time course experiment, indicating that the pKF707 *bph*BC-positive population was $<5 \times 10^5$ colony forming units.

After 1 week and 8 weeks of 2-CB enrichment, 100 individual colonies were picked from dilution plates and restreaked onto 1/2 strength R2A plates. None of the colonies isolated after 1 week of 2-CB enrichment hybridized with the pKF707 *bph*BC or pDA251 *bph*C gene probes. One percent of the colonies isolated after 8 weeks of 2-CB enrichment probed positive with pKF707 *bph*BC, and 16% probed positive with pDA251 *bph*C. However, none of these isolates produced the yellow *meta*-cleavage product from 2,3-dihydroxybiphenyl.

*Biodegradation of PCBs in TVA soil*

PCBs in the TVA soil were degraded only after the addition of a PCB-degrading strain (*Alcaligenes eutrophus* GG4202) and biphenyl (10 000 p.p.m.) Table 1, Fig. 4). After 32 days of incubation, the amount of PCBs was reduced from 40 p.p.m. (± 3 p.p.m.) (wt. PCB/wt soil) to 17 p.p.m. (± 1 p.p.m.). The pattern of congener degradation in the soil reflected the degradative abilities of *A. eutrophus* GG4202 as determined by previous resting cell assays [27]. No degradation was observed with biphenyl amendment in the absence of an exogenous strain (Fig. 4).

## DISCUSSION

Molecular diagnostics and chemical analysis provided mutually supporting results relative to characterization of PCB-degrading populations and PCB biodegradation in the TVA and New England soil. These approaches indicated that the bacterial populations in the TVA and the New England soil differed in their ability to degrade PCBs. 2-chlorobiphenyl, rather than biphenyl, was chosen as the substrate for enrichment of PCB-degrading bacteria from these soils for several reasons. First, previous attempts with enrichment of the TVA soil with 100 p.p.m. biphenyl, and 100 p.p.m. 4-

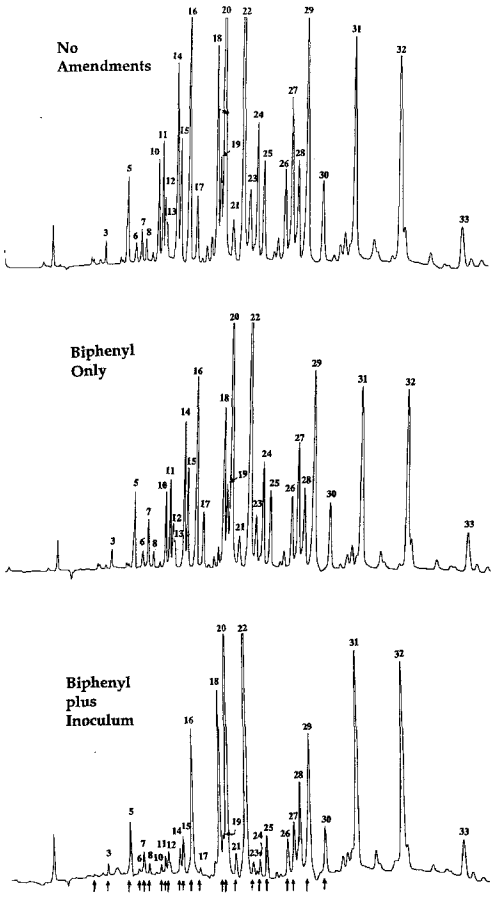



Fig. 4. Chromatograms of PCBs present in unamended TVA soil, TVA soil with 10 000 p.p.m. biphenyl, and TVA soil with 10 000 p.p.m. biphenyl and *A. eutrophus* GG4202 after 32 days incubation. Arrows indicate greater than 20% removal.

chlorobiphenyl were unsuccessful [27]. Second, the PCB present in the TVA soil is extensively weathered and originally reported to be Aroclor 1242. Many of the PCB congeners missing contain a chlorine at the 2 position. Therefore, microbes present in this soil might have a preference for 2-CB over biphenyl. Third, 2-chlorobiphenyl may be more selective for PCB-degrading bacteria. Several bacteria capable of degrading a large number of PCB congeners such as *Pseudomonas* LB400 and *A. eutrophus* H850, grow on 2-CB [6,17]. Not all bacteria capable of growing on biphenyl degrade a large number of PCB congeners [5,17].

The New England soil contained an active PCB-degrading bacterial population. These bacteria were identified by their rapid growth on 2-CB, their positive hybridization with the *bph*BC gene probes from pKF707 and C14-15, and the production of the yellow *meta*-cleavage product from 2,3-dihydroxybiphenyl. These bacterial colonies did not hybridize to the pDA251 *bph*BC gene probe. Previous experiments indicate that the *bph*BC gene from pKF707 and pDA251 do not cross hybridize at high stringency [27], suggesting that these gene probes may detect different biphenyl-degrading populations. The relationship between PCB congener degradation and the degradative ability of the indigenous strain NE2-1, the production of $^{14}C$-$CO_2$ from $^{14}C$-2CB, and the production of aqueous acidic metabolites (chlorobenzoates) indicated that PCB biodegradation, rather than abiotic losses, occurred in the soil.

In contrast, an active PCB-degrading population was not present in the TVA soil. Bacterial growth was not stimulated by the addition of biphenyl or 2-CB. After prolonged incubation (8 weeks) with 2-CB, bacteria that hybridized to the pDA251 *bph*BC gene probe were found. However, these bacteria did not produce the yellow *meta*-cleavage product from 2,3-dihydroxybiphenyl and did not grow on biphenyl or chlorobiphenyl. Chemical analyses did not indicate PCB-degradative activity.

Comparison of colony hybridization results with the three different *bph* gene probes indicated the importance of using multiple probes for site assessment and choosing the appropriate probes for process monitoring. The pKF707 *bph*BC and C14-15 *bph*AB gene probes hybridized to the PCB-degrading organisms in the New England soil, but the pDA251 *bph*C probe did not. Stimulation of growth of pKF707 *bph*BC-positive organisms by 2-CB amendment, and the similarity of congener degradation patterns between isolate NE2-1 and the treated soil, suggest that the most important PCB-degrading populations in this study were detected using the pKF707 *bph*BC gene probe. Other PCB-contaminated soils may contain a greater genetic diversity [39], so a wide array of gene probes including those for the Gram-positive biphenyl-degrading bacteria [3] may be required for effective bioremediation process monitoring. In the TVA soil, a few isolates probed positive with the pDA251 *bph*C gene probe but did not grow on biphenyl or chlorobiphenyl. From the standpoint of PCB bioremediation, these organisms can be considered to be false positives.

The number of PCB-degrading bacteria in the New England soil could be enumerated by hybridization of the pKF707 *bph*BC gene probe to either individual colonies or DNA soil extracts. Both methods indicated the same relative increase in *bph* gene sequences in soil enrichment experiments. However, the estimated number of *bph* sequences was 2–3 orders of magnitude higher than the number of *bph*-positive organisms. In PAH-contaminated soils using the naphthalene gene probe (*nah*A) the estimated number of *nah* sequences extracted from soils was 1–2 orders of magnitude higher than the number of *nah*-positive bacteria as measured by colony hybridization [32]. This may be due to nonculturable or nonviable cells. The direct DNA extraction method may prove useful for routine monitoring of test sites because it requires less time and materials, and the detection limit for biphenyl genes can be improved with the polymerase chain reaction [8]. Colony hybridizations have the advantage that phenotypic characteristics of the individual isolates on the agar plates can also be examined.

Molecular diagnostics may be useful in determining the factors limiting degradation in particular soils. Probing of microbial populations in TVA soil indicated a lack of indigenous PCB-degrading organisms. Addition of an exogenous PCB-degrading strain (*Alcaligenes eutrophus* GG4202) and 10 000 p.p.m. biphenyl resulted in >50% PCB degradation [27]. These results verify that cometabolic PCB degradation

was primarily limited by the lack of competent strains, rather than by soil toxicity or PCB bioavailability. In contrast, stimulation of PCB biodegradation in the New England soil only required inorganic nutrients and a cometabolic substrate. Since the TVA soil appears devoid of organisms that probe positive with the pKF707 *bph*BC gene probe, this probe is expected to be useful in monitoring the growth of *A. eutrophus* GG4202 or other exogenous PCB-degrading strains. Gene probes for tracking organisms may be especially valuable in cases where multiple exogenous strains with different congener degradation specificities are difficult to distinguish by other means.

Gene probes can be used for the detection and quantification of contaminant-degrading microbial populations or degradative genes. However, detection of DNA sequences does not verify that the microbial populations are active, and for cometabolic processes, growth of competent strains does not necessarily imply contaminant degradation. At least in the initial phases of an investigation, the ability of the gene probes to detect the relevant microbial population needs to be verified, and contaminant degradation must be confirmed by chemical analyses. Other molecular diagnostic techniques including messenger RNA probing and quantification [12] and biosensors using reporter strains [20] can provide further evidence of degradative activity. Site-specific molecular approaches to process monitoring developed at laboratory scale should aid in the verification of process performance in field scale bioremediation where many traditional chemical analyses become technically problematic.

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